

BU

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12Q 1/68, C12P 19/34, C07H 21/00, C07K 5/00		A1	(11) International Publication Number: WO 94/28171 (43) International Publication Date: 8 December 1994 (08.12.94)
(21) International Application Number: PCT/GB94/01174 (22) International Filing Date: 31 May 1994 (31.05.94)		(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KG, KP, KR, KZ, LK, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report.	
(30) Priority Data: 9311386.8 2 June 1993 (02.06.93) GB			
(71) Applicant (<i>for all designated States except US</i>): PNA DIAGNOSTICS A/S [DK/DK]; Lerso Parkalle 42, DK-2100 Copenhagen 0 (DK).			
(72) Inventor; and (75) Inventor/Applicant (<i>for US only</i>): STANLEY, Christopher, John [GB/GB]; 12 A Cromwell Place, St. Ives, Huntingdon, Cambridgeshire PE17 4JB (GB).			
(74) Agent: SMART, Peter, John; W.H. Beck, Greener & Co., 7 Stone Buildings, Lincoln's Inn, London WC2A 3SZ (GB).			
<p>(54) Title: NUCLEIC ACID ANALOGUE ASSAY PROCEDURES</p> <p>(57) Abstract</p> <p>An assay procedure to quantify the amount of starting template in a nucleic acid amplification procedure such as PCR involves trying the amplification in the presence of varying amounts of a nucleic acid analogue which in sufficient quantity will inhibit said amplification procedure by selective interaction with a nucleic acid species present, e.g. a PNA as described in WO-02-20703. The amount of template is given by the amount of nucleic acid analogue critical to cause failure of said amplification procedure.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

NUCLEIC ACID ANALOGUE ASSAY PROCEDURES

The present invention relates to assay procedures involving nucleic acid analogues by which quantitative or 5 semi-quantitative determinations of such analogues or nucleic acid sequences to which they hybridise can be made.

Nucleic acid amplification techniques are now in widespread use. These include the "PCR" (polymerase chain reaction) procedure described in EP-A-0200362 and EP-A-10 0201184, which is the technique in most widespread use, but also the "LCR" (ligase chain reaction) described in EP-A-0320308, the so-called "NASBA" or "3SR" technique which is described in "Proc. Natl. Acad. Sci. USA" Vol. 87 pp 1874-1878 March 1990 and "Nature" Vol. 350, No. 6313. pp 91-92 7th March 15 1991 and the "SDA" method described in "Nucleic Acid Research", Vol. 20 pp 1691-1696.

A number of strategies now exist to obtain quantitative information from amplification systems such as the polymerase chain reaction. In the simplest procedure the amount of 20 amplified DNA produced by the test sample is compared directly with a set of standard reactions prepared with known amounts of the same DNA. A linear relationship has been shown to exist between the amount of DNA present in the sample and the amount of amplification product eventually formed in the PCR 25 assay. However, the exponential nature of the PCR process means that minor differences in amplification efficiency between the sample tube and the separate tubes containing the known amounts of DNA will lead to large and unpredictable differences in the final product yield (Gilliland et al (1990) 30 "Proc. Natl Acad Sci. USA" 87, 2725-2729). Such direct comparisons between the amount of PCR product from the sample and standards tubes are therefore fundamentally flawed. A number of alternative procedures have been proposed to overcome this problem. These include the use of limiting 35 dilution of the sample (Thang et al (1991) "AIDS", 5,678-681) or the co-amplification of internal or external standards

(Kellogg et al (1990) "Anal. Biochem." 189, 202-208). The limiting dilution method is a very simple strategy that employs a Poisson distribution analysis of positive reactions to calculate the amount of the target sequence in the sample.

5 The limiting dilution method has a fundamental limitation because the linear relationship that exists between the initial amount of template DNA in the sample and the amount of amplification product obtained is only maintained for amounts of starting DNA within a limited range. Hence the

10 method is very imprecise when samples containing highly variable amounts of target DNA are examined. The alternative methods of co-amplification can provide a more reliable approach, in that they rely on the measurement of the ratio of target DNA and a co-amplified standard within the same

15 tube. However, they still suffer from a number of difficulties. The co-amplification of internal standards, such as ubiquitously expressed genes that are also present in the sample DNA is limited in its usefulness by the difference in amplification efficiency that exists between target and

20 reference sequences. This will affect the relative amounts of the products obtained in an uncontrollable manner. The alternative method of co-amplification of an external template possessing a similar length and the same primer recognition sequences as the target DNA is the most successful strategy

25 so far for a quantitative PCR assay (Gilliland et al "Proc. Natl. Acad. Sci. USA" 87, 2725-2729) but it still has three major disadvantages. The first problem is that the product from the target DNA and the co-amplified reference standard must be separated, usually by gel electrophoresis, and the

30 separated products must be quantified to determine the ratio of the products. Usually, 4 reactions or more are carried out at different dilutions of the external competitor template and the products from all these reactions must be scanned to provide a standard curve. The external competitive PCR

35 approach is therefore restricted to measurement methods that are capable of separating different PCR fragments and this

introduces further complexity and cost to the procedure. The second factor that restricts the usefulness of the external competitive PCR approach is that it cannot be used if the PCR products are to be detected directly without a prior 5 separation e.g. in a commonly used procedure biotin labelled PCR products are captured by hybridising to a capture probe immobilised to a microwell surface and subsequently detected by an avidin enzyme conjugate probe, leading to colour generation in the well. Distinguishing between control and 10 test samples in this type of assay requires the use of additional wells and different capture probes. The third disadvantage of the external competitive PCR approach is that the external standard must be designed carefully to have exactly the same amplification characteristics as the target 15 DNA from the sample. This is not easy to achieve and it requires considerable skill and expense to construct such an external standard and each assay must have a new external standard which greatly increases the cost of assay development.

20 WO-A-93/23706 discloses that certain nucleic acid analogues capable of hybridising with high affinity to nucleic acids can be used to inhibit nucleic acid amplification procedures such as PCR. The preferred nucleic acid analogues are described in WO-A-92/20703.

25 It has now been appreciated that the dependence of the successful inhibition of such nucleic acid amplification procedures on the amount of nucleic acid analogue present provides a means for deriving information regarding the amount of a nucleic acid which is present in a sample or about the 30 amount of a nucleic acid analogue which is present.

Accordingly, the present invention provides an assay procedure comprising attempting to perform a nucleic acid amplification procedure in the presence of a nucleic acid analogue which in sufficient quantity will inhibit said 35 amplification procedure by selective interaction with a nucleic acid species present, wherein the amount of said

-4-

nucleic acid or the amount of said nucleic acid analogue but not both is known or later otherwise determined, determining the success, failure, or degree of success of said amplification procedure and thereby deriving information 5 regarding the amount of said nucleic acid species or nucleic acid analogue present.

In most cases it is envisaged that it will be said nucleic acid species which is present in unknown quantity and this is preferably a template for said amplification.

10 The procedure preferably further comprises repeating said attempted assay procedure one or more times using one or more different amounts of said nucleic acid analogue or one or more dilutions of the sample so as to derive information regarding the relative amounts thereof needed to be present to inhibit 15 said amplification procedure.

Preferably, a calibration curve is established by carrying out the same amplification procedure using a series of known relative amounts of the nucleic acid species and the nucleic acid analogue.

20 Thus using known amounts of said nucleic acid one may establish a calibration curve of amplification product quantity at a particular nucleic acid template concentration against nucleic acid analogue concentration. One may then carry out the assay procedure on a sample containing an 25 unknown amount of the nucleic acid, preferably simultaneously conducting a number of assay runs at different dilutions of the nucleic acid analogue to identify the dilution between which the amplification procedure moves from success to failure. This will produce a semi-quantitative estimate of the amount of nucleic acid initially present, as the quantity 30 of nucleic acid analogue required for inhibition of the amplification will be proportional to the quantity of nucleic acid template present. Thus if one finds that a calibration curve based on x ng of plasmid moves from successful amplification to failure at a $y \mu\text{M}$ concentration of nucleic acid analogue, a sample requiring a $2y \mu\text{M}$ concentration of nucleic 35

-5-

acid analogue for inhibition will contain approximately 2x ng of template.

There are a number of important advantages provided by this new procedure which we refer to as quantitation by inhibition (QI). Firstly, the QI process is not a ratio approach and so it is not necessary to seek an internal standard for amplification, or to laboriously construct an external standard. In a QI PCR process, for example, the nucleic acid analogue can be directed against the primers and this is very simple to achieve since the sequences are already known. The QI process does not produce two products which must be separated and quantitated as must be done in the external standard approach. Hence, the QI process is well suited to the more favoured methods of product detection such as hybridisation in a microplate well. The QI process does not require any dilution of the sample and no complex mathematical treatment of the results is required. The QI process is therefore far superior to the limiting dilution method. The QI process is largely unaffected by variations in amplification efficiencies in individual tubes since the results are scored by comparison to a threshold level alone.

The QI process is well suited to a semi-quantitative measurement. The dynamic range can be very large, or very small depending on the concentration range of nucleic acid analogue chosen. The QI process can employ a single concentration of inhibitor to give a simple YES/NO answer, or many concentrations in multiple wells to provide a near quantitative result. The imprecision of the QI process, in terms of misclassification of sample DNA concentration, is a function of the variation in the product amplification process at the threshold point only.

The nucleic acid analogue is preferably capable of hybridising to a nucleic acid of complementary sequence to form a hybrid which is more stable against denaturation by heat than a hybrid between the conventional deoxyribo-

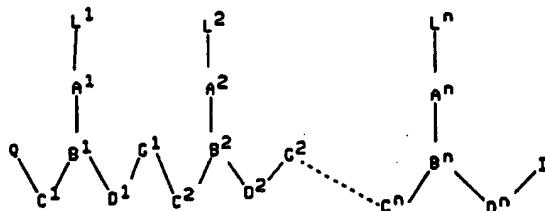
-6-

nucleotide corresponding in sequence to said analogue and said nucleic acid.

Preferably, said nucleic acid analogue is a peptide nucleic acid in which said backbone is a polyamide backbone, 5 each said ligand being bonded directly or indirectly to an aza nitrogen atom in said backbone, and said ligand bearing nitrogen atoms mainly being separated from one another in said backbone by from 4 to 8 intervening atoms.

Preferably also, the nucleic acid analogue is capable of 10 hybridising to a double stranded nucleic acid in which one strand has a sequence complementary to said analogue, in such a way as to displace the other strand from said one strand.

Preferably, the nucleic acid analogue has the general formula 1:



Formula 1

15

wherein:

n is at least 2,

each of L¹-Lⁿ is independently selected from the group consisting of hydrogen, hydroxy, (C₁-C₄) alkanoyl, naturally 20 occurring nucleobases, non-naturally occurring nucleobases, aromatic moieties, DNA intercalators, nucleobase-binding groups, heterocyclic moieties, and reporter ligands, but normally at least one L will be a nucleobase binding group such as a naturally occurring nucleobase and preferably at 25 least 90% of the groups L will be such nucleobase binding groups;

WO 94/28171

-7-

each of C¹-Cⁿ is (CR⁶R⁷)_y where R⁶ is hydrogen and R⁷ is selected from the group consisting of the side chains of naturally occurring alpha amino acids, or R⁶ and R⁷ are independently selected from the group consisting of hydrogen, 5 (C₁-C₆) alkyl, aryl, aralkyl, heteroaryl, hydroxy, (C₁-C₆) alkoxyl, (C₁-C₆) alkylthio, NR³R⁴ and SR⁵, where R³ and R⁴ are as defined below, and R⁵ is hydrogen, (C₁-C₆) alkyl, hydroxy, alkoxy, or alkylthio-substituted (C₁ to C₆) alkyl or R⁶ and R⁷ taken together complete an alicyclic or heterocyclic

10 system;

each of D¹-Dⁿ is (CR⁶R⁷)_z where R⁶ and R⁷ are as defined above;

each of y and z is zero or an integer from 1 to 10, the sum y + z being from 2 to 10 (preferably being more than 2, and most preferably each of y and z being 1 or 2;

15 each of G¹-Gⁿ⁻¹ is -NR³CO-, -NR³CS-, -NR³SO- or -NR³SO₂-;

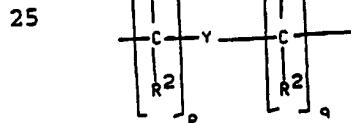
in either orientation, where R³ is as defined below:

each of A¹-Aⁿ and B¹-Bⁿ are selected such that:

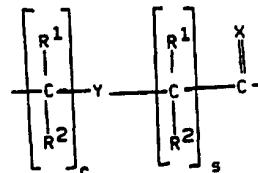
(a) A is a group of formula (IIa), (IIb), (IIc) or

(IId), and B is N or R³N⁺; or

20 (b) A is a group of formula (IId) and B is CH;



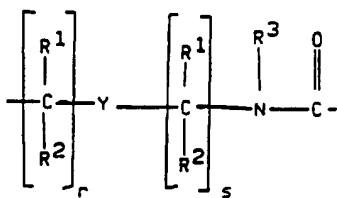
30 Formula IIa



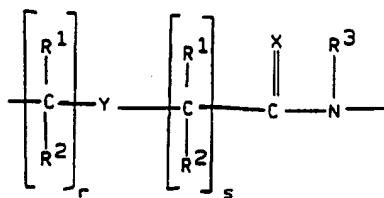
Formula IIb

- 8 -

5



10



Formula IIc

Formula IIId

15

wherein:

X is O, S, NR³, CH₂ or C(CH₃)₂;

Y is a single bond, O, S or NR⁴;

each of p and q is zero or an integer from 1 to 5, the
20 sum p+q being not more than 10;

each of r and s is zero or an integer from 1 to 5, the
sum r+s being not more than 10;

each R¹ and R² is independently selected from the group
consisting of hydrogen, (C₁-C₄)alkyl which may be hydroxy- or
25 alkoxy- or alkylthio-substituted, hydroxy, alkoxy, alkylthio,
amino and halogen; and

each R³ and R⁴ is independently selected from the group
consisting of hydrogen, (C₁-C₄)alkyl, hydroxy- or alkoxy- or
alkylthio-substituted (C₁-C₄)alkyl, hydroxy, alkoxy, alkylthio
30 and amino;

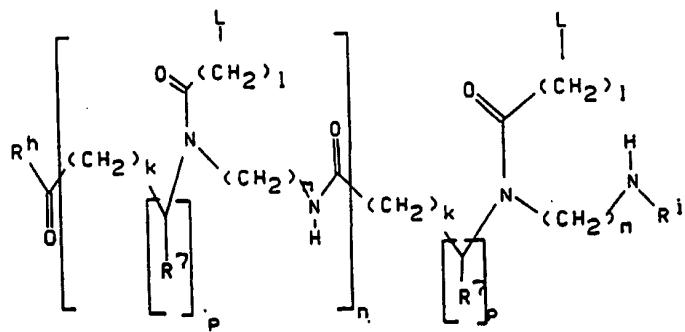
Q is -CO₂H, -CONR'R'', -SO₃H or -SO₂NR'R'' or an
activated derivative of -CO₂H or -SO₃H; and

I is -NR'R'', where R' and R'' are independently selected
from the group consisting of hydrogen, alkyl, amino protecting
35 groups, reporter ligands, intercalators, chelators, peptides,
proteins, carbohydrates, lipids, steroids, nucleosides,

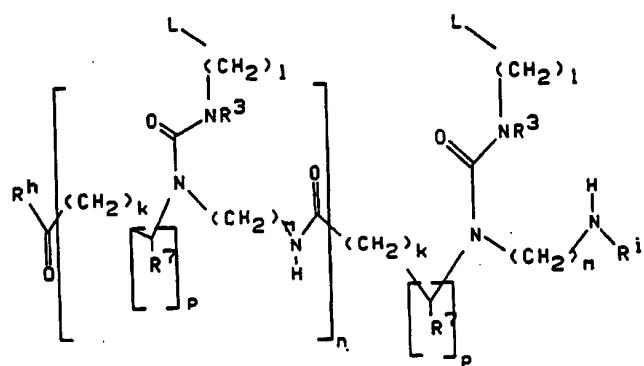
- 9 -

nucleotides, nucleotide diphosphates, nucleotide triphosphates, oligonucleotides, including both oligoribonucleotides and oligodeoxyribonucleotides, oligonucleosides and soluble and non-soluble polymers.

5 More preferably, said nucleic acid analogue comprises a compound of the general formula III, IV or V:

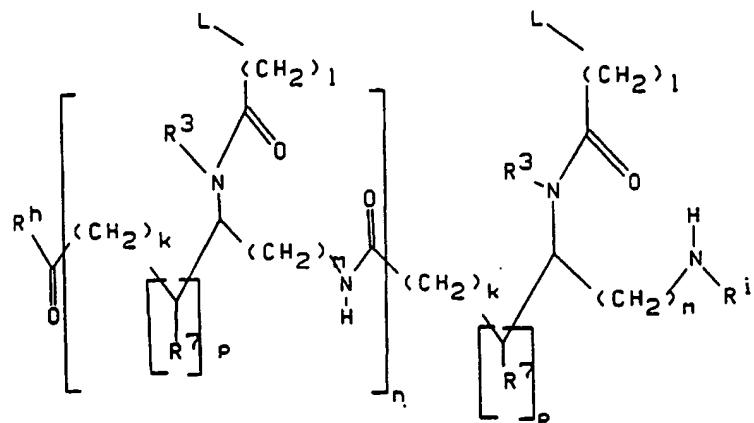


Formula III



Formula IV

-10-



Formula V

wherein:

- 5 each L is independently selected from the group consisting of hydrogen, phenyl, heterocyclic moieties, naturally occurring nucleobases, and non-naturally occurring nucleobases;
- 10 each R⁷ is independently selected from the group consisting of hydrogen and the side chains of naturally occurring alpha amino acids;
- n is an integer greater than 1,
- each k, l, and m is, independently, zero or an integer from 1 to 5;
- 15 each p is zero or 1;
- R^h is OH, NH₂ or -NHLysNH₂; and
- Rⁱ is H or -COCH₃.
- Techniques for the synthesis of preferred nucleic acid
- 20 analogues are to be found in WO-A-92/20703.

Example 1

The PNA T₁₀-LysNH₂ was synthesised as in Egholm, M., Buchardt, O., Nielsen, P.E. and Berg, R.H. (1992) "J. Amer. Chem. Soc." 114, 1895-1897 and Egholm, M., Buchardt O., Nielsen, P.E. and Berg, R.H. (1992) "J. Amer. Chem. Soc." 114, 9677-9678. The plasmid pT10KS was constructed by cloning the complementary oligonucleotides 5'-GATCCT₁₀G and 5'-GATCCA₁₀G into respective strands at the BamH I site of the Bluescript 5 KS+plasmid (Stratagene). The control plasmid pCKS was constructed by cloning a 99bp PCR fragment (with no target sites for the PNA used in this example) into the SmaI site of the bluescript KS+plasmid. Using standard techniques plasmids were isolated from selected clones of recombinant E. Coli 10 JM103, purified by buoyant density centrifugation in CsCl 15 gradients and sequenced by the dideoxy method.

The following oligonucleotide primers were used in the PCR reactions: reverse primer (5'-CACACAGGAAACAGCTATGAC-SEQ1) and forward primer (5'-GTAAAACGACGGCCAGT-SEQ2). PCR 20 amplifications were carried out in a 50 µl volume containing 1 ng of each plasmid, 0.2 µM of each primer, 200 µM dNTP, 10 mM Tris-HCl, pH 8.3 (at 25°C), 10 mM KCl, and 3 mM MgCl₂.

The concentrations of the PNA employed were 0 µM, 13.2 µM, 6.6 µM, 3.2 µM, 1.6 µM, 0.8 µM, 0.4 µM and 0.2 µM.

25 The PCR reactions were overlain with 2 drops of paraffin oil and incubated at 90°C for 2 minutes before the amplification process was initiated by the addition of 3U of Stoffel polymerase (Perkin Elmer Cetus). A LEP amplifier machine (IgG Biotech) was used in all experiments and standard 30 PCR cycle profiles were 96°C, 2 min - 55°C, 3 min - 35°C, 1 min - 65°C, 2 min - 35 cycles.

To ensure that the formation of the PNA/DNA complexes preceded PCR primer binding and extension the normal 3 step 35 PCR cycle was expanded with a distinct PNA annealing step at 55°C a temperature well above the T_m of the PCR primers.

-12-

Products resulting from the PCR process were separated by gel electrophoresis and stained using ethidium bromide using standard techniques. A Polaroid[™] photograph was taken of the stained gel and the intensity of the bands was assessed
5 by visual inspection.

Table 1 shows the result of PCR inhibition in the presence of increasing amounts of PNA T₁₀-LysNH₂. The two plasmid templates described above were used; namely the pT10KS plasmid which directs the amplification of a 246bp fragment
10 containing an A₁₀ target site and the control plasmid, pCKS, which directs the amplification of a 329bp non-target fragment.

The ability to clamp the PCR reaction is indicated using the following nomenclature: ++ = PCR product; + = some PCR
15 product and - = no PCR product; NT = not tested.

TABLE 1

Plasmid	Concentrations of PNA (μ M)							
	0	0.2	0.4	0.8	1.6	3.2	6.6	13.2
p T10KS	++	++	++	++	+	-	-	-
pCKS	NT	NT	NT	NT	NT	NT	NT	++

When the PNA T₁₀-LysNH₂ is either absent or present in a concentration equal to or below 1.6 μ M the pT10KS plasmid directs the synthesis of the expected 246bp PCR fragment. At concentration at or about 3.2 μ M PNA T₁₀-LysNH₂, however, no product is produced. The absence of product is not due to a
25 non-specific inhibitory effect of PNA T₁₀-LysNH₂ on the PCR reaction, since even at the highest concentration used (13.2 μ M), PNA T₁₀-LysNH₂ will not inhibit the amplification of the expected 329bp fragment from the pCKS control plasmid. This,
30 it is seen that the PNA T₁₀-LysNH₂ can prevent the amplification of its cognate target DNA in a sequence specific
35

-13-

manner. The concentration dependence of inhibition is quite steep.

Virtually no inhibition is observed at 0.8 μ M PNA while more than 75% inhibition takes place at 1.6 μ M and total 5 inhibition is seen at 3.2 μ M PNA.

Taking a sample containing an unknown quantity of the pT10KS plasmid, one may repeat the above procedure seeking to find a dilution of the sample plasmid at which the profile of inhibition using the concentrations of PNA used above matches 10 the results shown in Figure 1, so establishing the concentration of plasmid.

More preferably however, one may simply calculate the quantity of template by finding the critical quantity of PNA in μ M required to inhibit amplification and relying on 15 proportionality between this and the amount of template DNA in ng by a factor of 1 ng/3.2 μ M derived from the results above.

Example 2

20

Plasmid and primers used in this example are as follows:

Plasmid p62-1

The plasmid was constructed in two steps. First the 25 complementary oligonucleotides (5'-GATCCTGTACGTACAACTA-3' - SEQ3 and 5'-GATCTAGTTGTGACGTACAG-3' -SEQ4) comprising the recognition sequence for PNA62 (H-TGTACGTACAACTA-Gly-NH₂) were cloned into the BamHI site of the plasmid pUC19 to yield p62. Second, a 556bp PstI/HindIII fragment of E.coli phase 30 λ genome was cloned into the PstI/HindIII site of p62 to yield p62-1.

Primers

Two primers were synthesised with the following sequence: 35 reverse primer 5'-GAAACAGCTATGAC-3'-SEQ5 and forward primer 5'-TGTACGTACAACTA-3'-SEQ6 (the sequence of the forward primer is identical to the sequence of the PNA62). In a PCR reaction

-14-

the forward and reverse primer together with the p62-1 plasmid direct the amplification of a 659bp fragment.

PCR reactions were set-up containing either 1ng, 5ng or 25ng of plasmid p62-1, reverse and forward PCR primers and various concentrations of PNA 62. Each PCR reaction (50 µl) contained 200 µM dNTP, 10mM Tris-HCl pH 9.9 (20°C), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatine, 0.1% Triton X-100, 1U supertaq enzyme (Stratagene), 10 pmol of each of the two PCR primers and PNAs and p 62-1 plasmid as indicated below in Table 2. PCR conditions were 96°C 2 min, 65°C 2 min, 35°C 30 sec, 70°C 2 min - 30 cycles. The ability to clamp the PCR reaction is indicated using the following nomenclature: + = PCR product; += some PCR product and - = no PCR product.

15

TABLE 2

		PNA Concentration					
		None	1.63µM	325nM	65nM	13nM	2.6nM
	1ng plasmid	++	-	-	-	++	++
20	5ng plasmid	++	-	-	+	++	++
	25ng plasmid	++	-	+	++	++	++

When 1ng of plasmid was used the concentration of PNA that effectively clamped the PCR reaction was found to be 65nM. When the amount of plasmid target was increased five fold (from 1 to 5 ng) the PNA concentration that was required for efficient clamping was proportionally increased to 325 nM. Similarly, increasing the initial plasmid target 25 fold required a 25 fold increase in PNA (1.63 µM) for efficient clamping to take place. Thus, the QI or PCR clamp method can be used to quantitate the initial amount of target in the reaction.

Had the quantitative plasmid content of the sample containing 5 ng been unknown, it could readily have been

deduced from the results obtained at 1 ng of plasmid, used as a calibration curve, and the cut-off figure of 65 nM found for effective clamping of the amplification of the unknown (5 ng plasmid) sample.

5 It should be noted that, in Example 1 and Example 2, only one nucleic acid analogue is employed and this is directed against one PCR primer site only. Hence a linear amplification process driven by the non-inhibited primer will take place in these experiments. Such linearly amplified material will not be detected by the gel electrophoretic method described here because of sensitivity limitations. To achieve total inhibition of a PCR process it is necessary to employ 10 two nucleic acid molecules directed at either both primers or at both strands of the double stranded nucleic acid target. 15 This will be important if more sensitive methods of detection than gel electrophoresis/staining by ethidium bromide are to be employed.

Whilst the invention has been described with reference to the preferred embodiments illustrated by the Examples above, many variations and modifications are possible within 20 the scope of the invention.

Also, the invention includes a kit for use in quantifying an amount of target nucleic acid in a sample, comprising reagents for use in an amplification procedure to amplify a 25 sequence in said nucleic acid, and at least two non-complementary nucleic acid analogue sequences each for hybridising to a sequence to be amplified respectively in a strand of said target nucleic acid and a nucleic acid strand complementary thereto to prevent amplification thereof.

30 The nucleic acid sequences referred to above are detailed in the following listing.

-16-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT:

- (A) NAME: PNA Diagnostics A/S
- (B) STREET: Lerso Park Alle 42
- (C) CITY: Copenhagen
- 10 (E) COUNTRY: Denmark
- (F) POSTAL CODE (ZIP): DK-2100

10

(ii) TITLE OF INVENTION: Nucleic Acid Analogue Assay
Procedures

15

(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
 - 20 (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (EPO)

25

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: GB 9311386.8
- (B) FILING DATE: 02-JUN-1993

30

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

35

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

-17-

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

5 (iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

10

CACACAGGAA ACAGCTATGA C

21

(2) INFORMATION FOR SEQ ID NO: 2:

15

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- 20 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

25

(iii) ANTI-SENSE: NO

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GTAAAACGAC GGCCAGT

17

35 (2) INFORMATION FOR SEQ ID NO: 3:

WO 94/28171

-18-

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

10

(iii) ANTI-SENSE: NO

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GATCCTGTAC GTCACAACTA

20

20 (2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA (genomic)

30

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

-19-

GATCTAGTTG TGACGTACAG

20

(2) INFORMATION FOR SEQ ID NO: 5:

5

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 14 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

15

(iii) ANTI-SENSE: NO

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GAAACAGCTA TGAC

14

25 (2) INFORMATION FOR SEQ ID NO: 6:

30

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 15 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

35

(iii) HYPOTHETICAL: NO

-20-

(iii) ANTI-SENSE: NO

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TGTACGTCAC AACTA

15

10

15

-21-

CLAIMS

1. An assay procedure comprising attempting to perform a nucleic acid amplification procedure in the presence of a nucleic acid analogue which in sufficient quantity will inhibit said amplification procedure by selective interaction with a nucleic acid species present, wherein the amount of said nucleic acid or the amount of said nucleic acid analogue but not both is known or later otherwise determined, determining the success, failure, or degree of success of said amplification procedure and thereby deriving information regarding the amount of said nucleic acid species or nucleic acid analogue present.
- 15 2. An assay procedure as claimed in Claim 1, wherein said nucleic acid species is present in unknown quantity and is a template for said amplification.
3. An assay procedure as claimed in Claim 2, further comprising repeating said attempted assay procedure one or more times using one or more different amounts of said nucleic acid analogue so as to derive information regarding the amount thereof needed to be present to inhibit said amplification procedure.
4. An assay procedure as claimed in any preceding claim, wherein said nucleic acid analogue hybridises to a primer hybridisation site of said amplification procedure.
- 10 5. An assay procedure claimed in any preceding claim, wherein the nucleic acid analogue comprises a polymeric strand which includes a sequence of ligands bound to a polyamide, polythioamide, polysulphinamide or polysulphonamide backbone made up of linked backbone moieties, which analogue is capable of hybridisation to a nucleic acid of complementary sequence.

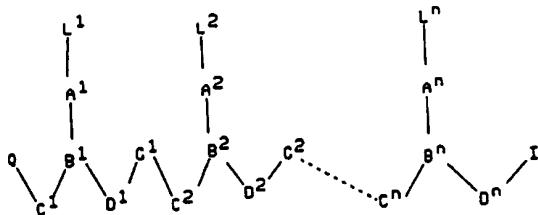
-22-

6. An assay procedure as claimed in any preceding claim,
wherein the nucleic acid analogue is capable of hybridising
to a nucleic acid of complementary sequence to form a hybrid
which is more stable against denaturation by heat than a
5 hybrid between the conventional deoxyribo-nucleotide
corresponding in sequence to said analogue and said nucleic
acid.

7. An assay procedure as claimed in any preceding claim,
10 wherein said nucleic acid analogue is a peptide nucleic acid
in which said backbone is a polyamide backbone, each said
ligand being bonded directly or indirectly to an aza nitrogen
atom in said backbone, and said ligand bearing nitrogen atoms
mainly being separated from one another in said backbone by
15 from 4 to 8 intervening atoms.

8. An assay procedure as claimed in any preceding claim,
wherein the nucleic acid analogue is capable of hybridising
to a double-stranded nucleic acid in which one strand has a
20 sequence complementary to said analogue, in such a way as to
displace the other strand from said one strand.

9. An assay procedure as claimed in any preceding claim,
wherein the nucleic acid analogue has the general formula 1:



Formula 1

wherein:

- n is at least 2,
each of L¹-Lⁿ is independently selected from the group
5 consisting of hydrogen, hydroxy, (C₁-C₄) alkanoyl, naturally
occurring nucleobases, non-naturally occurring nucleobases,
aromatic moieties, DNA intercalators, nucleobase-binding
groups, heterocyclic moieties, and reporter ligands;
- each of C¹-Cⁿ is (CR⁶R⁷)_y where R⁶ is hydrogen and R⁷ is
10 selected from the group consisting of the side chains of
naturally occurring alpha amino acids, or R⁶ and R⁷ are
independently selected from the group consisting of hydrogen,
(C₂-C₆) alkyl, aryl, aralkyl, heteroaryl, hydroxy, (C₁-
C₆) alkoxy, (C₁-C₆) alkylthio, NR³R⁴ and SR⁵, where R³ and R⁴
15 are as defined below, and R⁵ is hydrogen, (C₁-C₆) alkyl,
hydroxy, alkoxy, or alkylthio-substituted (C₁ to C₆) alkyl or
R⁶ and R⁷ taken together complete an alicyclic or heterocyclic
system;
- each of D¹-Dⁿ is (CR⁶R⁷)_z where R⁶ and R⁷ are as defined
20 above;
- each of y and z is zero or an integer from 1 to 10, the
sum y + z being from 2 to 10;
- each of G¹-Gⁿ⁻¹ is -NR³CO-, -NR³CS-, -NR³SO- or -NR³SO₂-,
in either orientation, where R³ is as defined below;
- 25 each of A¹-Aⁿ and B¹-Bⁿ are selected such that:
(a) A is a group of formula (IIa), (IIb), (IIc) or

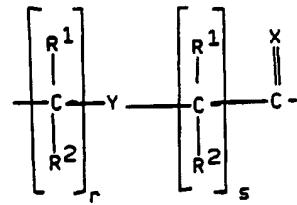
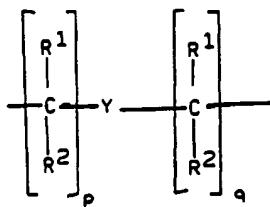
-24-

(IIId), and B is N or R^3N^+ ; or

(b) A is a group of formula (IIId) and B is CH;

5

10

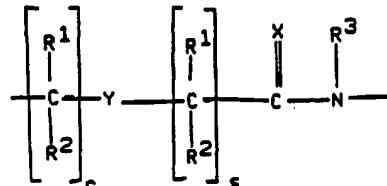
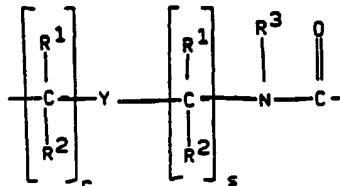


Formula IIa

Formula IIb

15

20



25

Formula IIc

Formula IID

30 wherein:

X is O, S, Se, NR³, CH₂ or C(CH₃)₂;Y is a single bond, O, S or NR⁴;

each of p and q is zero or an integer from 1 to 5, the sum p+q being not more than 10;

35 each of r and s is zero or an integer from 1 to 5, the sum r+s being not more than 10;

-25-

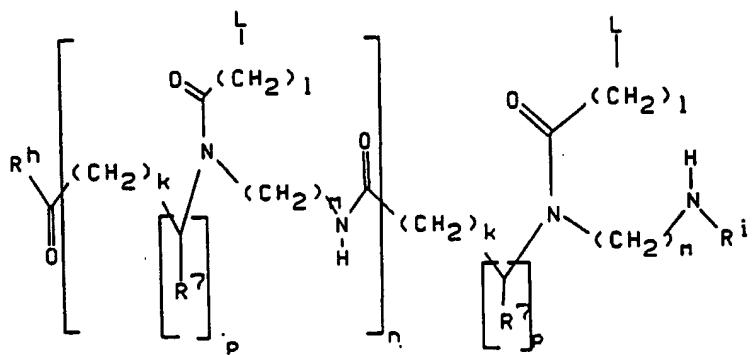
each R¹ and R² is independently selected from the group consisting of hydrogen, (C₁-C₄)alkyl which may be hydroxy- or alkoxy- or alkylthio-substituted, hydroxy, alkoxy, alkylthio, amino and halogen; and

5 each R³ and R⁴ is independently selected from the group consisting of hydrogen, (C₁-C₄)alkyl, hydroxy- or alkoxy- or alkylthio-substituted (C₁-C₄)alkyl, hydroxy, alkoxy, alkylthio and amino;

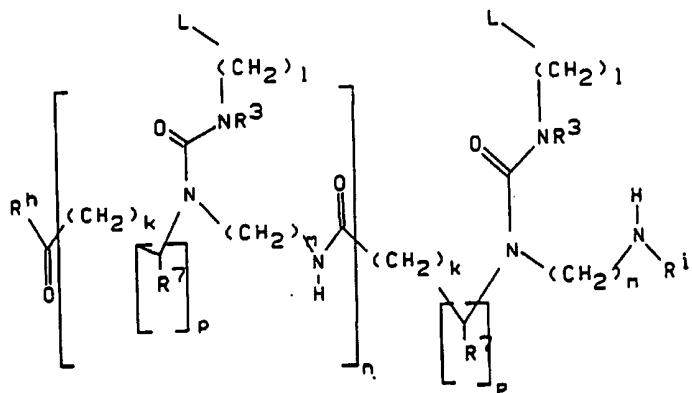
Q is -CO₂H, -CONR'R'', -SO₃H or -SO₂NR'R'' or an activated derivative of -CO₂H or -SO₃H; and

10 I is -NR'R'', where R' and R'' are independently selected from the group consisting of hydrogen, alkyl, amino protecting groups, reporter ligands, intercalators, chelators, peptides, proteins, carbohydrates, lipids, steroids, nucleosides, 15 nucleotides, nucleotide diphosphates, nucleotide triphosphates, oligonucleotides, including both oligoribonucleotides and oligodeoxyribonucleotides, oligonucleosides and soluble and non-soluble polymers.

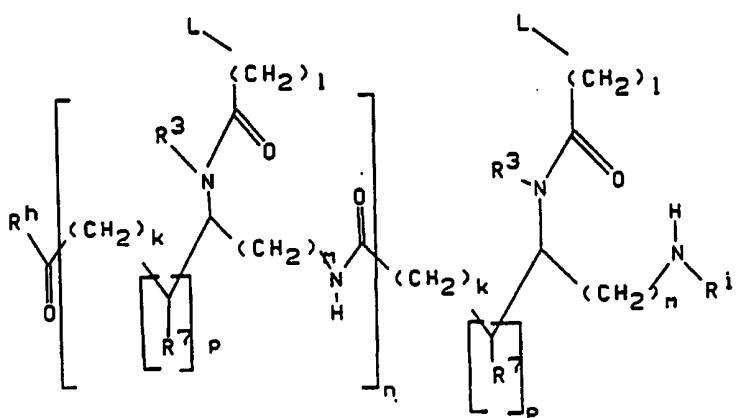
20 10. An assay procedure as claimed in Claim 9, wherein said nucleic acid analogue comprises a compound of the general formula III, IV or V:



Formula III



Formula IV



Formula V

wherein:

- 5 each L is independently selected from the group consisting of hydrogen, phenyl, heterocyclic moieties, naturally occurring nucleobases, and non-naturally occurring nucleobases;

each R⁷ is independently selected from the group consisting of hydrogen and the side chains of naturally occurring alpha amino acids;

n is an integer greater than 1,

5 each k, l, and m is, independently, zero or an integer from 1 to 5;

each p is zero or 1;

R^h is OH, NH₂ or -NHLysNH₂; and

Rⁱ H or COCH₃.

10

11. A kit for use in quantifying an amount of target nucleic acid in a sample, comprising reagents for use in an amplification procedure to amplify a sequence in said nucleic acid, and at least two non-complementary nucleic acid analogue sequences each for hybridising to a sequence to be amplified respectively in a strand of said target nucleic acid and a nucleic acid strand complementary thereto to prevent amplification thereof.

20

12. A kit as claimed in Claim 11, wherein said kit further comprises at least two amplification primers and said nucleic acid analogue sequences are such as to compete with said primers to hybridise to said target nucleic acid.

25

13. A kit as claimed in Claim 11, wherein said kit further comprises at least two amplification primers and said nucleic acid analogue sequences are such as to hybridise to said target nucleic acid between primer binding sites therein.

INTERNATIONAL SEARCH REPORT

International application No. PCT/GB 94/01174
--

A. CLASSIFICATION OF SUBJECT MATTER IPC 5 C12Q1/68	C12P19/34	C07H21/00	C07K5/00
---	-----------	-----------	----------

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	WO,A,93 25706 (BUCHARDT ET AL.) 23 December 1993 see the whole document	1-10
A	WO,A,92 20703 (BUCHARDT ET AL) 26 November 1992 cited in the application see the whole document	1-10
A	WO,A,91 02817 (CETUS CORP.) 7 March 1991	
A	WO,A,92 01812 (CEMU BIOTECHNIK AB) 6 February 1992	
	-/-	

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- 'A' document defining the general state of the art which is not considered to be of particular relevance
- 'E' earlier document but published on or after the international filing date
- 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- 'O' document referring to an oral disclosure, use, exhibition or other means
- 'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

1

Date of the actual completion of the international search

Date of mailing of the international search report

1 September 1994

21-09-1994

Name and mailing address of the ISA
European Patent Office, P.B. 5818'Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl
Fax (+31-70) 340-3016

Authorized officer

Osborne, H

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 94/01174

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 87 , April 1990 , WASHINGTON US pages 2725 - 2729 GILLILAND, G. EZ AL. 'analysis of cytokine mRNA and DNA: Detection and quantitation by competitive polymerase chain reaction' ----	

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/GB 94/01174

Patent document cited in search report	Publication date		Patent family member(s)	Publication date
WO-A-9325706	23-12-93	AU-B-	4323593	04-01-94
WO-A-9220703	26-11-92	AU-A- AU-A- CA-A- WO-A- EP-A- EP-A-	1880692 1884392 2109320 9220702 0586474 0586618	30-12-92 30-12-92 25-11-92 26-11-92 16-03-94 16-03-94
WO-A-9102817	07-03-91	US-A- AU-A- CA-A- EP-A- JP-T-	5219727 6352390 2064906 0497784 5504886	15-06-93 03-04-91 22-02-91 12-08-92 29-07-93
WO-A-9201812	06-02-92	AU-B- AU-A- CA-A- EP-A-	648330 8238691 2087982 0540585	21-04-94 18-02-92 25-01-92 12-05-93